



## Colloids and Surfaces B: Biointerfaces

journal homepage: [www.elsevier.com/locate/colsurfb](http://www.elsevier.com/locate/colsurfb)Elucidation of the mechanisms of action of Bacteriophage K/nano-emulsion formulations against *S. aureus* via measurement of particle size and zeta potentialPatricia Perez Esteban<sup>a,b</sup>, A. Toby A. Jenkins<sup>b</sup>, Tom C. Arnot<sup>a,\*</sup><sup>a</sup> Department of Chemical Engineering, University of Bath, Claverton Down, Bath BA2 7AY, UK<sup>b</sup> Department of Chemistry, University of Bath, Claverton Down, Bath BA2 7AY, UK

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## ABSTRACT

In earlier work we have demonstrated the effect that nano-emulsions have on bacterial growth, and most importantly the enhanced bacteriophage infectivity against *Staphylococcus aureus* in planktonic culture when phage are carried in nano-emulsions. However, the mechanisms of enhancement of the bacteriophage killing effect are not specifically understood. This work focuses on the investigation of the possible interactions between emulsion droplets and bacterial cells, between emulsion droplets and bacteriophages, and finally interactions between all three components: nano-emulsion droplets, bacteria, and bacteriophages. The first approach consists of simple calculations to determine the spatial distribution of the components, based on measurements of particle size. It was found that nano-emulsion droplets are much more numerous than bacteria or bacteriophage, and due to their size and surface area they must be covering the surface of both cells and bacteriophage particles. Stabilisation of bacteriophages due to electrostatic forces and interaction with nano-emulsion droplets is suspected, since bacteriophages may be protected against inactivation due to 'charge shielding'. Zeta potential was measured for the individual components in the system, and for all of them combined. It was concluded that the presence of nano-emulsions could be reducing electrostatic repulsion between bacterial cells and bacteriophage, both of which are very negatively 'charged'. Moreover, nano-emulsions lead to more favourable interaction between bacteriophages and bacteria, enhancing the anti-microbial or killing effect. These findings are relevant since the physicochemical properties of nano-emulsions (i.e. particle size distribution and zeta potential) are key in determining the efficacy of the formulation against infection in the context of responsive burn wound dressings—which is the main target for this work.

Data Access: All data created during this research are openly available from the University of Bath data archive at <http://doi.org/10.15125/BATH-00166>.

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## 1. Introduction

Bacteriophages are being reconsidered as therapeutic agents against bacterial infection, due to the persistence and evolution of antibiotic resistant organisms worldwide [19]. Delivery of bacteriophages to the point of infection, especially in topical applications, requires an appropriate and biocompatible vehicle. Several examples of encapsulation or stabilisation of bacteriophages for therapeutic purposes can be found in the literature [18,26,2,3,17,24,11]. We have used PIT nano-emulsions in order to efficiently store bacteriophages and formulate a topical cream [5]. Nano-emulsions have recently been considered as suitable substi-

tutes of micro-emulsions in cosmetic and healthcare applications [14,8]. We have demonstrated that stabilisation of bacteriophages using PIT nano-emulsions produced enhanced infectivity of bacteriophages against *Staphylococcus aureus* infections in planktonic culture. We have also observed that the presence of different concentrations of nano-emulsion droplets produced variations in bacterial growth patterns. The mechanisms that cause such variations, especially in the lytic activity of bacteriophage, are unknown and very little information can be found in the literature. One reference suggests that the presence of emulsion droplets produces higher bacteriophage titres [10] but it does not explore the reasons behind such an increase in bacteriophage proliferation. We suggested that introduction of nano-emulsions leads to stabilisation of bacteriophages due to electrostatic forces, i.e. 'charge shielding', and enhanced interaction between the droplets and bacteria and bacteriophage. Further investigation of this system is needed,

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## Nomenclature

$A_{\text{droplet}}$	Surface areas of emulsion droplets (cm <sup>2</sup> )
$C_{\text{droplets}}$	Concentration of droplets (droplets ml <sup>−1</sup> )
CFU ml <sup>−1</sup>	Colony forming units per millilitre
DLS	Dynamic light scattering
$\varepsilon$	Dielectric constant (–)
$f(ka)$	Henry's function (–)
MOI	Multiplicity of infection
MRSA	Methicillin resistant <i>Staphylococcus aureus</i>
$\eta$	Viscosity (cP)
$n_{\text{droplets}}$	Number of droplets (–)
PdI	Polydispersity index
PIT	Phase inversion temperature (°C)
PFU ml <sup>−1</sup>	Plaque forming units per millilitre
$r$	Radius of an emulsion droplet (cm)
RO	Reverse osmosis
SD	Standard deviation
TSB	Tryptic Soy broth
$u_E$	Electrophoretic mobility (μm cm V <sup>−1</sup> s <sup>−1</sup> )
$V$	Volume (ml)
$V_{\text{droplet}}$	Volume of one single emulsion droplet (cm <sup>3</sup> , or ml)
$V_{\text{emulsion}}$	Total volume of nano-emulsion (ml)
$V_{\text{oil}}$	Volume of oil that was added to the nano-emulsion (ml)
Z	Zeta potential (mV)

mainly due to the novelty of the work and the lack of supporting evidence in the literature. Elucidation of the mechanisms in this complex system is important for the optimisation of the antimicrobial formulation, which is intended for use in responsive burn wound dressings. We suspect that the spatial distribution of the components (i.e. bacterial cells, phages and nano-droplets) is key; thus, Transmission Electron Microscopy (TEM) and Cryo-TEM were tested in order to determine the exact positioning and relative arrangement of the components. However, these techniques were not successful: the sample treatment that is required for TEM of bacteriophages destroyed the nano-emulsion droplets, which were therefore not visible. Measurements of size and zeta potential are presented for each of the components individually, for paired combinations, and finally for all of the elements together: bacteria, bacteriophages and nano-emulsion droplets. We again use *S. aureus* as the model organism for most of the experimental tests due to its relevance as a ubiquitous pathogen in burn wounds [21]. Zeta potential and size measurements of bacteriophages and bacteria have been previously used in the literature in order to study phage adsorption in different ionic strength conditions [27].

## 2. Materials and methods

### 2.1. Chemicals

Brij® O10 (Polyoxyethylene (10) oleyl ether), soybean oil, tryptic soy broth (TSB), NaCl, MgSO<sub>4</sub>·7H<sub>2</sub>O, Tris–Cl, and gelatin were purchased from Sigma–Aldrich (Dorset, UK). Reverse Osmosis water was produced in the laboratory via membrane filtration of tap water.

### 2.2. Bacterial and bacteriophage strains

*S. aureus* strains H560, H325, and Btn766, and Bacteriophage K were obtained from AmpliPhi Biosciences (Bedfordshire, UK). Usual stock culture methods were employed.

### 2.3. Emulsification method

The thermal phase inversion emulsification method was used [23,6] to formulate nano-emulsions with the following composition: 5% (w/w) soybean oil as the organic phase, 15% (w/w) Brij® O10 as surfactant, and 80% (w/w) SM buffer (100 mM NaCl, 8 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 50 mM Tris–Cl, 0.002% (w/v) gelatin, pH 7.5) as the aqueous phase. The experimental protocol is identical to that in our previous paper [5]. The concentration of surfactant exceeds the critical micellar concentration [9] but the existence of nano-emulsion droplets in this system has been previously reported via microscopy [13].

### 2.4. Size measurement

The measurements of size were performed using the ZETASIZER Nano Series ZSP (Malvern Instruments) via dynamic light scattering (DLS). For nano-emulsion droplets this was done at 25 °C by diluting 100 μl of the freshly prepared formulation into 10 ml of deionised water. For all strains of *S. aureus*, 100 μl of overnight bacterial culture was diluted into 10 ml of deionised water, so the concentration of bacteria was approximately 10<sup>6</sup> CFU ml<sup>−1</sup>. This concentration is equivalent to nearly 0.1 optical absorbance units (a.u.) at 600 nm. The same experimental conditions were used for the measurement of Bacteriophage K, by diluting 100 μl of bacteriophage stock suspension (previously filtered with a 0.22 μm filter) into 10 ml of deionised water. Bacteriophage K suspensions had a phage concentration of about 10<sup>5</sup>, 10<sup>6</sup> and 10<sup>7</sup> PFU ml<sup>−1</sup>. Independent triplicates of each set of samples were measured, and each measurement consisted of 5 runs using the DLS instrument. The peak values of particle diameter (nm) and the polydispersity index (PdI) for the different samples were obtained.

### 2.5. Zeta potential measurement

Zeta potential of suspended particles can be defined as the difference in potential between the bulk of the conducting medium in which they are dispersed, and the stationary layer of fluid surrounding the suspended particle. Zeta potential is closely related to the surface charge of colloidal particles, and provides an indication of the stability of colloidal systems. It is heavily affected by pH, and therefore also ionic strength. Due to the external charge of particles, when they are exposed to an external electrical field, they will migrate towards an electrode of the opposite charge at a certain velocity. This velocity is the electrophoretic mobility of the particle, the magnitude of which is directly measured. Zeta potential can be calculated using the Henry equation [4]:

$$u_E = \frac{2\varepsilon z f(ka)}{3\eta} \tag{1}$$

where  $u_E$  is the electrophoretic mobility (μm cm V<sup>−1</sup> s<sup>−1</sup>),  $\varepsilon$  is the dielectric constant,  $z$  is the zeta potential (mV),  $f(ka)$  is the Henry's function, and  $\eta$  is the viscosity (cP). Electrophoretic determinations of zeta potential are made in aqueous media and at moderate electrolyte concentrations. The value of  $f(ka)$  is 1.5, and this is referred to as the Smoluchowski approximation [22]. It is worth noting that the Smoluchowski approximation and the use of the Henry's function involve the assumption of spherical particles. Henry's equation can be modified for spherical particles covered with a polymer layer. This is the case of emulsion droplets and bacterial cells, which are presumably covered by a layer of surfactant (Polyoxyethylene (10) oleyl ether), and can be considered soft particles, or soft bio-colloids in the case of bacteria [7]. The general electrophoresis theory that applies to soft particles covered with a layer of ion-penetrable uncharged polymer involves a modified Henry's function  $f(ka, kb, \lambda a, \lambda b)$ , which also depends on the radius

**Table 1**Volume, concentration, surface area and concentration ratios in the system formed by PIT emulsion droplets, *S. aureus* cells, and Bacteriophage K.

Component	Average $V$ (ml)	Average concentration (ml <sup>-1</sup> )	Average surface area (cm <sup>2</sup> )	$V_{\text{component}}/V_{\text{droplets}}$	$C_{\text{droplets}}/C_{\text{component}}$
PIT nano-emulsion droplets	$3 \times 10^{-18}$	$1.6 \times 10^{16}$ droplets	$1 \times 10^{-11}$	1.0	1.0
<i>S. aureus</i> cells	$3 \times 10^{-13}$	$1.0 \times 10^6$ CFU	$2 \times 10^{-8}$	$1 \times 10^5$	$2 \times 10^{12}$
Bacteriophage K	$2 \times 10^{-15}$	$1.0 \times 10^5$ PFU	$8 \times 10^{-10}$	$7 \times 10^2$	$2 \times 10^{13}$

of the colloidal covered particle  $b$  and the hydrodynamic softness  $1/\lambda$  [16]. Considering the values of hydrodynamic softness for bacteria in [7], the sizes determined via DLS measurements and the assumption that the polymer layer has a thickness comparable to that of emulsion droplets, values for  $f(ka, kb, \lambda a, \lambda b)$  can be estimated from Ref. [16], and incorporated into the modified Henry's function to calculate the apparent zeta potential from the measured electrophoretic mobility. In addition to this, bacteriophages can be considered as cylindrical colloidal particles [15], and Henry's equation for bacteriophages oriented at an arbitrary angle between their axes and the applied electric field is:

$$\mu_E = \frac{\epsilon Z}{3\eta} [1 + 2f(ka)] \quad (2)$$

Measurement of electrophoretic mobility was performed using the ZETASIZER Nano Series ZSP (Malvern Instruments). Unless stated otherwise, all measurements were carried out at 25 °C by diluting 100  $\mu$ l of sample in 10 ml of the deionised water. Triplicates of each sample were measured, and each measurement comprised 10–100 runs, depending on the need to find a stable reading. The pH of the samples was  $7.0 \pm 0.2$  in all cases.

### 3. Results and discussion

#### 3.1. Initial investigation of the mechanisms

Particle sizes (diameters) were determined via the DLS technique. PIT nano-emulsion droplets are spherical, as are *S. aureus* cells. The only assumption that was made in these preliminary calculations is that bacteriophages are also spherical (although their morphology is more complex), with their diameter corresponding approximately to the length of their head plus their tail. This assumption is not completely arbitrary, since it provides an indication of the space that bacteriophages might occupy regardless of their orientation. The average radius of PIT nano-emulsion droplets was found to be around 9 nm; DLS measurements and TEM micrographs [1] indicate that the 'radius' of Bacteriophage K (assuming it is spherical) was around 80 nm; finally, the average radius of *S. aureus* H560 was found via DLS measurement to be about 425 nm.

The estimated number of emulsion droplets in a sample can be calculated as the total volume of oil that was used to formulate the nano-emulsion, divided by the volume of one droplet (Eq. (3) as described by Eq. (4)). The concentration of emulsion droplets per millilitre is simply the number of emulsion droplets (calculated in Eq. (4)), divided by the total volume of nano-emulsion prepared

$$V_{\text{droplet}} = \frac{4}{3}\pi r^3 \quad (3)$$

where  $V_{\text{droplet}}$  is the volume of one single emulsion droplet (cm<sup>3</sup>, or ml), and  $r$  is the radius of the emulsion droplet (cm). Eq. (2)

can also be applied to calculate the estimated volume occupied by bacteriophages and *S. aureus* cells using their corresponding radii.

The number of droplets in the PIT nano-emulsion formulation in this work (5% (w/w) soybean oil as the organic phase, 15% (w/w) Brij® O10 as surfactant, and 80% (w/w) SM buffer) is shown in Eq. (4), taking into account that 5 ml of oil was added for the emulsion preparation.

$$n_{\text{droplets}} = \frac{V_{\text{oil}}}{V_{\text{droplet}}} = \left( \frac{5\text{ml}}{\frac{4}{3}\pi r^3} \right) \cong 1.6 \times 10^{18} \text{ droplets} \quad (4)$$

where  $n_{\text{droplets}}$  is the number of droplets (dimensionless),  $V_{\text{oil}}$  is the volume of oil that was added to the nano-emulsion (ml),  $V_{\text{droplet}}$  is the volume of a single droplet (ml), and  $r$  is the droplet radius (cm). This result corresponds to 5 ml of oil used in the preparation, with an average droplet radius of 9 nm. From this, the concentration of emulsion droplets per millilitre in the 100 ml nano-emulsion was found to be  $1.6 \times 10^{16}$  droplets ml<sup>-1</sup>.

Finally, if surface areas are to be compared, the assumption that bacteriophages are spherical is applied again.

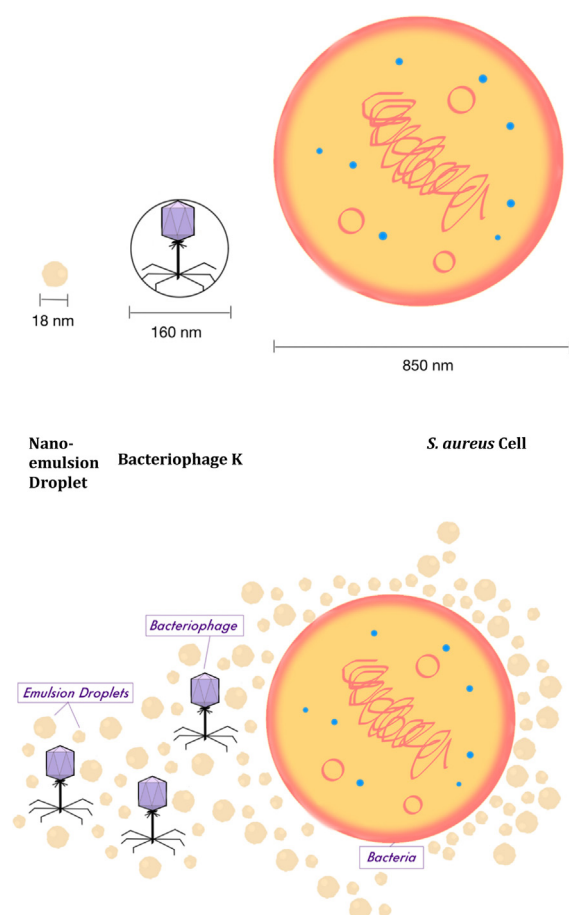
For an initial bacterial concentration of approximately  $10^6$  CFU ml<sup>-1</sup>, the initial concentration of bacteriophage is  $10^5$  PFU ml<sup>-1</sup> (MOI = 0.1), and the concentration of droplets per millilitre is  $1.6 \times 10^{16}$  droplets ml<sup>-1</sup>. Table 1 shows a summary of the key parameters that might give an idea of the spatial distribution and interactions of the components in the system.

Based only on size/volume/surface area data, it is spatially impossible that bacteriophages are encapsulated by, or sitting on the surface of, the nano-emulsion droplets. The same must be true for the bacterial cells. Bacteriophage K (see Table 1) is a hundred times more voluminous than a nano-emulsion droplet, and a single cell of *S. aureus* is a hundred thousand times bigger than a nano-emulsion droplet. Based on these values, the most probable situation is that both bacteria and bacteriophage are surrounded or covered or shielded by a large amount of nano-emulsion droplets. This conclusion is supported by the concentration ratios: as seen in Table 1 there are approximately one billion nano-emulsion droplets per bacterial cell, and ten billion nano-emulsion droplets per bacteriophage particle. This is shown schematically in Fig. 1.

In addition to these preliminary calculations, the literature indicates that *S. aureus* cells are generally hydrophobic [20], and hence it is expected that bacteriophages that infect *S. aureus* will also be hydrophobic. This depends on the hydrophobicity of their environment since their structures are basically proteins [25]. The polarity of cells and bacteriophages can suggest that bacterial cells and phage particles will attract oil droplets (i.e. they are lipophilic), further confirming the likely spatial distribution of the elements in the system. It is clear that encapsulation of bacteriophages inside the PIT nano-emulsion droplets can be ruled out. Therefore, the next step comprises investigation of the mechanisms of interaction within the system.

**Table 2**Droplet size distribution and zeta potential of different *S. aureus* strains. Average values of three repeats are presented, together with  $\pm$  standard deviation ( $N = 3$ ).

Strain	PdI	Size (nm)	Zeta potential (mV)	Mobility ( $\mu\text{m cm V}^{-1} \text{s}^{-1}$ )
<i>S. aureus</i> H560	$0.952 \pm 0.084$	$949 \pm 161$	$-297.0 \pm 8.2$	$-3.105 \pm 0.092$
<i>S. aureus</i> H325	$0.760 \pm 0.374$	$881 \pm 167$	$-255.0 \pm 6.0$	$-2.662 \pm 0.065$
<i>S. aureus</i> Btn766	$0.301 \pm 0.080$	$734 \pm 138$	$-260.3 \pm 5.2$	$-2.723 \pm 0.055$



**Fig. 1.** Comparison of volumes of PIT nano-emulsion droplet, Bacteriophage K, and *S. aureus* cell (left to right) (A), and most likely spatial distribution of the components of the composite anti-microbial system (B). There is a layer of surfactant surrounding all species (not shown for clarity).

### 3.2. Size and zeta potential of nano-emulsion droplets

PIT nano-emulsions were characterised in terms of their particle size distribution and zeta potential, and a single narrow peak was found at  $18.34 \pm 0.36$  nm. The polydispersity index (Pdl) was  $0.159 \pm 0.036$ , indicating that the sample is very monodispersed (the Pdl is close to zero); also the variability of particle size is small given the low standard deviation. The zeta potential of the nano-droplets was  $-0.59 \pm 0.61$  mV, and their electrophoretic mobility was  $0.046 \pm 0.048 \mu\text{m cm V}^{-1} \text{s}^{-1}$ . The interval of zeta potential and mobility values, together with their corresponding standard deviation, show that PIT nano-emulsion droplets are practically not charged at all (zeta potential  $\approx 0$  mV). This is expected, since the surfactant that was used is non-ionic, although the continuous phase is SM buffer, which contains salts and hence has ionic strength.

### 3.3. Size and zeta potential of bacterial cells

Measurement of size and polydispersity index of bacterial cells was performed using the DLS technique, and results are shown in Table 2. The diameters of all *S. aureus* strains are close to 1  $\mu\text{m}$ , i.e. much bigger than the size of PIT nano-emulsions. Their Pdl indicates that bacterial samples are generally quite polydispersed, with a broad size distribution. This is due to cells dividing and the presence of cells at different stages of the growth cycle. The measurements of size of *S. aureus* cells were acceptably accurate, given their spherical morphology. In order to determine if bacterial den-

sity should be taken into account in the measurement of size, the concentration of the three strains of *S. aureus* was increased by a factor of 4, and an increase in apparent size was observed, as shown in Fig. 2. Concentrated samples show diameters that are almost double the size of that found in the more diluted samples, as shown by comparing the non-shaded bars to the shaded bars in Fig. 2. Thus, a statistical analysis of the difference of averages was performed, between the average cell size of a particular strain and its average size for a concentration multiplied by four. The cell diameters were compared in terms of their average, using the *t*-test, included in the statistical software Origin 8<sup>®</sup>. From the statistical analysis, it can be concluded that the concentration of bacteria is a critical factor when measuring cell size in deionised water. It is likely that the lack of salts results in cell aggregation at the higher concentrations. Smaller concentrations of bacteria yield results that are comparable to values found using other techniques, such as electron microscopy. This is important in order to select the appropriate cell concentration for the measurement of zeta potential, where an aqueous suspension is preferred.

The zeta potential of the three bacterial strains is very similar, shown in Table 2, and very negative, with a small standard deviation. The value of  $f(ka, kb, \lambda a, \lambda b)$  for bacterial cells was found to be approximately 0.2 under identical assumptions to those of the Smoluchowski approximation [16]. The zeta potential of bacteria is much higher (in absolute terms) than that for the nano-emulsions, and therefore their electrophoretic mobility is also much higher. High absolute values of zeta potential (very positive or very negative) indicate stability with respect to coalescence, sedimentation, etcetera [12]. However, bacterial cells are very big, and gravitational forces might produce aggregation despite their electrostatic stability.

### 3.4. Size and zeta potential of Bacteriophage K

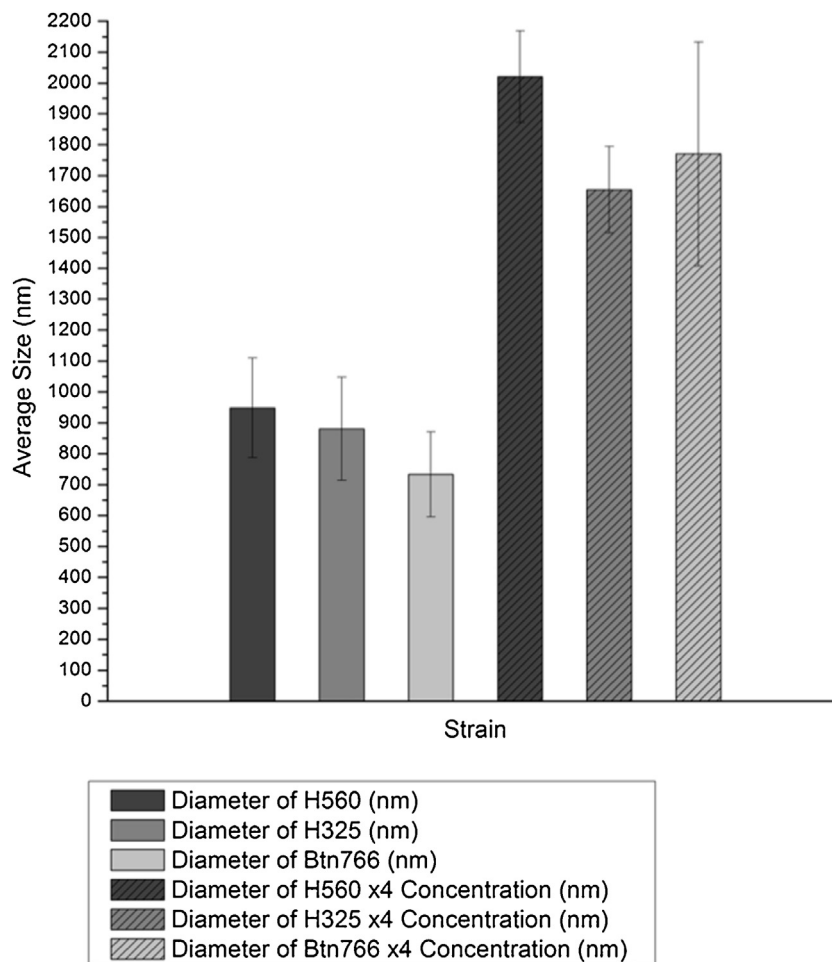
It should be remembered that Bacteriophage K is not spherical, and therefore the results obtained from DLS measurements correspond to the larger dimension of the phage (its length). Three different concentrations of Bacteriophage K were tested in order to determine whether the concentration of the sample is an important factor:  $10^5$ ,  $10^6$  and  $10^7$  PFU  $\text{ml}^{-1}$ . The measured size for the more diluted samples was found to be approximately 2.5 times the size of less diluted samples, suggesting aggregation—see Fig. 3. Bacteriophage K samples with a concentration of  $10^6$  PFU  $\text{ml}^{-1}$  have a size of  $154.8 \pm 5.1$  nm, with a Pdl of  $0.265 \pm 0.057$ . Bacteriophage samples are not as polydispersed as bacterial samples, suggesting that the size of the bacteriophages is more uniform. A concentration of  $10^6$  PFU  $\text{ml}^{-1}$  was selected for measurements of zeta potential, as their apparent size was in better agreement with TEM pictures [1].

Analysis of Bacteriophage K at a concentration of  $10^6$  PFU  $\text{ml}^{-1}$  gave a zeta potential of  $-17.4 \pm 1.7$  mV and an electrophoretic mobility of  $-1.091 \pm 0.115 \mu\text{m cm V}^{-1} \text{s}^{-1}$  according to Eq. (2) for cylindrical colloidal particles, and a value for  $f(ka, kb, \lambda a, \lambda b)$  of approximately 0.7 [16]. Bacteriophage K shows a negative zeta potential, but not as negative as *S. aureus*. This suggests that, apart from the obvious biochemical interaction between bacteriophages and the cell surface receptors, there is an electrostatic repulsion that could make contact between the two species less effective.

### 3.5. Size and zeta potential of bacterial cells + Bacteriophage K

The measurement procedure for size and zeta potential for *S. aureus* cells and Bacteriophage K in combination was identical to that for the separate components (Figs. 2 and 3 and Tables 2 and 3). It was thought that the measurement time and temperature (25 °C) would be neither long nor high enough, respectively, for the bacteriophages to bind bacterial cells to start their lytic cycle. The data for





**Fig. 2.** Summary of average sizes of *S. aureus* H560, H325 and Btn766 in deionised water. Error bars represent the standard deviation of triplicates ( $N=3$ ).

**Table 3**

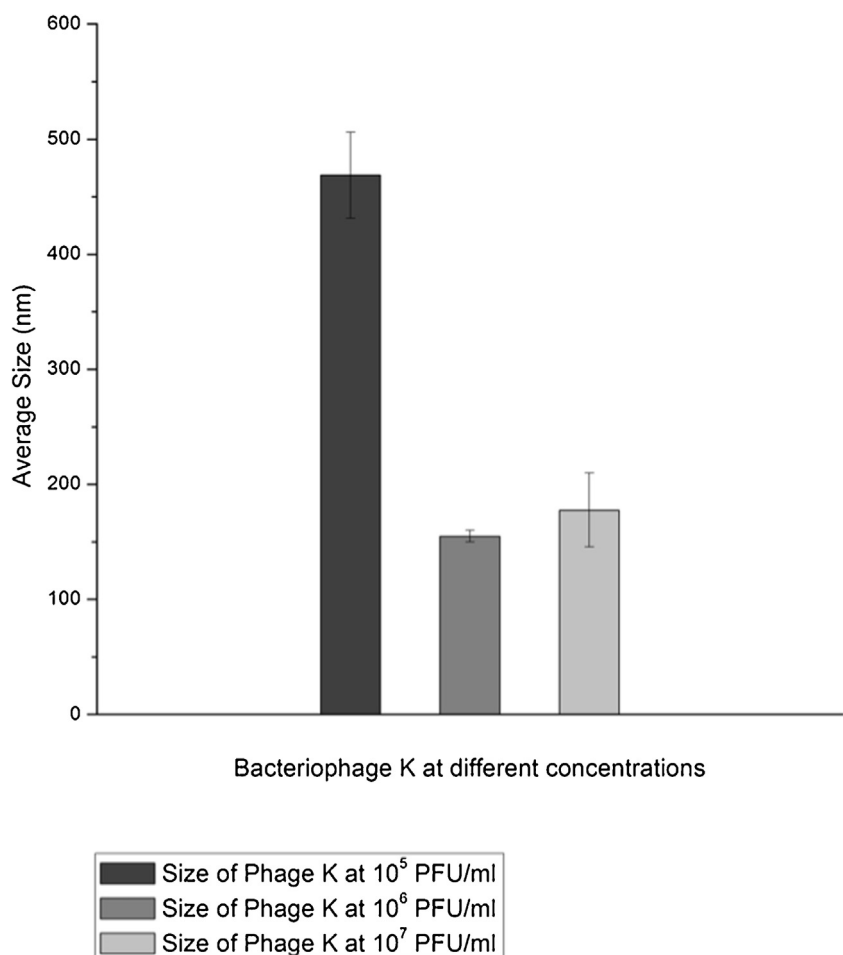
Droplet size ( $D$ ) and zeta potential ( $ZP$ ) distribution of different *S. aureus* strains + Bacteriophage K. Average values of three repeats, together with  $\pm$  standard deviation ( $SD$ ) ( $N=3$ ).

<i>S. aureus</i> H560 + Bacteriophage K			<i>S. aureus</i> H325 + Bacteriophage K			<i>S. aureus</i> Btn766 + Bacteriophage K		
Peak 1 (bacteria)								
Average $D$ (nm)		SD $D$ (nm)	Average $D$ (nm)		SD $D$ (nm)	Average $D$ (nm)		SD $D$ (nm)
2141		988	1014		391	1251		280
Peak 2 (Bacteriophage K)								
Average $D$ (nm)		SD $D$ (nm)	Average $D$ (nm)		SD $D$ (nm)	Average $D$ (nm)		SD $D$ (nm)
251.6		45.7	121.7		32.2	101.3		2.4
<i>S. aureus</i> H560 + Bacteriophage K			<i>S. aureus</i> H325 + Bacteriophage K			<i>S. aureus</i> Btn766 + Bacteriophage K		
Average $ZP$ (mV)		SD $ZP$ (mV)	Average $ZP$ (mV)		SD $ZP$ (mV)	Average $ZP$ (mV)		SD $ZP$ (mV)
−309.7		5.2	−305.2		11.2	−254.2		0.7

size and zeta potential are presented in Table 3. The concentration of all strains of bacteria and bacteriophages was  $10^6$  CFU  $ml^{-1}$  and  $10^6$  PFU  $ml^{-1}$  respectively. Two peaks were observed for each sample; they were completely separated (resolved), and the results are coherent with the independent measurements for the two species. An exception was *S. aureus* H560, which showed a very high value of cell size, indicating aggregation of cells. The fact that the size values are in nearly all cases very similar to those of the independent elements indicates that there is no binding while the measurements took place. This is significant regarding the zeta potential results as it allows for discernment between the biochemical inter-

actions (non-existent under these conditions) and the electrostatic interactions.

The zeta potential measurements were performed at 25 °C, by diluting 200  $\mu l$  of Bacteriophage K suspension of approximately  $10^6$  PFU  $ml^{-1}$  and 10  $\mu l$  of bacterial overnight culture, into 5 ml of deionised water. The results are presented in Table 3. The zeta potential for the combined system is slightly higher than that for bacterial samples alone (see Table 2), and it is clear that the overall zeta potential is predominantly influenced by the bacterial cells. Table 3 also shows very little variability in the data, as indicated by the small values of standard deviation ( $SD$ ).



**Fig. 3.** Summary of average sizes of Bacteriophage K in deionised water. Error bars represent the standard deviation of triplicates ( $N=3$ ).

**Table 4**  
Droplet size distribution of Bacteriophage K in PIT nano-emulsion formulations. Average values of three repeats, together with their  $\pm$  standard deviation ( $N=3$ ).

Bacteriophage K + PIT nano-emulsion	
Peak 1 (Nano-emulsion)	
Average D (nm)	SD D (nm)
16.80	0.20
Peak 2 (Bacteriophage K)	
Average D (nm)	SD D (nm)
269.7	10.1

### 3.6. Size and zeta potential of Bacteriophage K + PIT nano-emulsion droplets

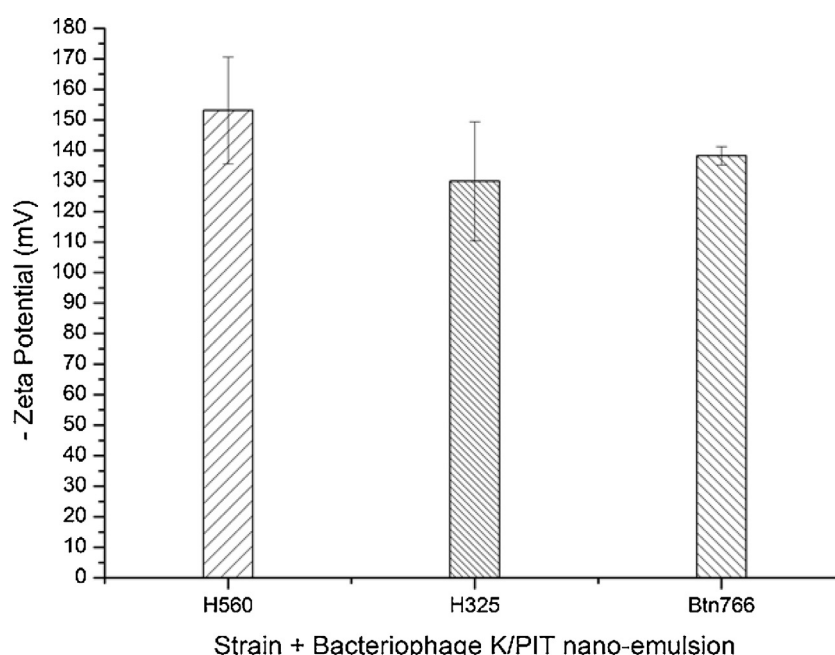
Determination of size and zeta potential of the Bacteriophage K/PIT nano-emulsion system could be significant in explaining the enhanced infectivity of these formulations. The measurement procedure was identical to that previously described, and the concentration of Bacteriophage K was  $10^6$  PFU ml<sup>-1</sup>. The size results are shown in Table 4 – the two peaks were well resolved, and the values are similar to the ones that were found when the species were measured independently (see Fig. 3).

The zeta potential value was  $-1.64 \pm 0.21$  mV. Clearly, the presence of nano-emulsions makes the zeta potential of Bacteriophage K much less negative (compared to  $-17.4 \pm 1.7$  mV for Bacteriophage K alone). The literature suggests that different environmental conditions may alter the zeta potential of bacteriophages due to electrostatic interactions [27]. This is a significant finding, and it provides a potential explanation as to why the bacteriophage/nano-

emulsion preparations are much more effective against bacteria. The electrostatic repulsion between two species that are highly negatively charged (bacteria and bacteriophage) is almost totally eliminated by the presence of the nano-emulsion droplets. This can be interpreted as follows: in the absence of nano-emulsions two different kinds of forces act in the system: firstly, a positive, attractive force that brings bacteria and bacteriophage together, such as the biochemical interaction between the bacteriophage tail fibres and the receptors on the bacterial cell surface; and secondly, a negative, repulsive force that separates bacteria and bacteriophage, purely via electrostatic repulsion. When PIT nano-emulsions are included, the electrostatic repulsion is eliminated, favouring the interaction between bacteriophages and bacteria in a much more efficient way. This is further confirmed in Section 3.7, where the zeta potential of all the components is measured in combination. Potentially, and as it has been assumed before, there is a layer of surfactant that covers or is adsorbed to the surface of the colloidal particles considered in this study, particularly to that of bacteria and bacteriophages since at concentrations above the critical micelle concentration, some surfactants can form complexes with lipophilic proteins. However, this has been taken into account in the zeta potential calculations using the modified Henry's function.

### 3.7. Zeta potential of bacterial cells + Bacteriophage K + PIT nano-emulsion droplets

Finally, zeta potential of the combined system (*S. aureus* cells + Bacteriophage K/PIT nano-emulsions) was measured using a



**Fig. 4.** Zeta potential distribution of *S. aureus* strains plus Bacteriophage K in PIT nano-emulsion formulations. Error bars represent the standard deviation of triplicates ( $N=3$ ).

concentration of bacteria and bacteriophage of  $10^6$  CFU ml $^{-1}$  and  $10^6$  PFU ml $^{-1}$  respectively, under the same experimental conditions as before. The results are shown in Fig. 4 for the three strains of *S. aureus* tested. The zeta potential of the complex mixtures was calculated considering that the contribution of bacterial cells and bacteriophage is equal, since their concentration is the same ( $10^6$  CFU ml $^{-1}$  and  $10^6$  PFU ml $^{-1}$  respectively).

The zeta potential is less negative than that for bacteria + Bacteriophage K, shown in Table 3, confirming the attenuated electrostatic repulsion between bacteria and bacteriophages due to the presence of the nano-emulsion droplets. The hypothesis of 'charge shielding' of bacteriophages via PIT nano-emulsions, has therefore been demonstrated, and the mechanism of enhanced infectivity has been explained.

#### 4. Conclusions

The main aim of this work was to study of the mechanisms and interactions a bacteria-emulsion-bacteriophage system which is intended for use as an antimicrobial formulation in burn wound dressings. The first relevant finding is the elucidation of the spatial distribution of the components in the system via simple calculations of size, volume, surface area and respective concentrations of bacteria, droplets and bacteriophages. It was found that encapsulation of bacteriophages was impossible due to spatial and size considerations, despite the apparent hydrophobicity of bacteriophages as stated in the literature [25], and that nano-emulsion droplets cover the surface of bacterial cells and are surrounding bacteriophages in a much higher number (approximately ten billion droplets per bacteriophage particle). This is relevant since it helps explain the enhanced infectivity of bacteriophage/nano-emulsion formulations against *S. aureus* infections. The most significant discovery in this study was the explanation of the mechanisms of enhanced infectivity via simple measurements of particle size distribution and zeta potential. It can be concluded that electrostatic interactions play a vital role in this system, and this is supported by the literature. Zeta potential values for Bacteriophage K/PIT nano-emulsion combinations compared to values for Bac-

teriophage K alone, suggest that the presence of nano-emulsions eliminates possible electrostatic repulsions between bacteria and bacteriophages, both of which are very negatively 'charged'. This leads to a more effective initial contact/interaction, and hence results in the enhanced killing effect of the bacteriophage formulations.

A study of the mechanisms and interactions in this complex system is important in relation to optimising the formulation for its role in responsive and anti-microbial wound dressings. Future work will explore the use of such formulations in more realistic wound environments, where bacteria are not generally in planktonic culture, but forming biofilms.

#### Conflict of interest

The authors declare that they have no conflicts of interest.

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